Thermal Modification of Chrysotile Asbestos: Evidence for Decreased Cytotoxicity

by R. Valentine,* M. J. W. Chang,* R. W. Hart,† G. L. Finch‡ and G. L. Fisher*

Many asbestiform minerals exhibit temperature-dependent thermoluminescence. Since thermoluminescence involves electronic transitions within crystalline materials, the effect of temperature on asbestos cytotoxicity was evaluated. Heat pretreatment of Canadian chrysotile asbestos reduces its cytotoxicity towards cultured human fibroblasts and bovine alveolar macrophages. When monitored 44 hr after the addition of either 200°C or 400°C heat-pretreated asbestos, alveolar macrophage viability was approximately 40% higher than comparable amounts of unheated asbestos. Similarly, asbestos toxicity, expressed as fibroblast growth inhibition, was inversely related to the asbestos pretreatment temperature in the following manner, 70°C > 200°C >400°C = unexposed fibroblast controls. Pretreatment of chrysotile asbestos to 400°C reduced its adsorptive capacity for bovine serum albumin by 25%. Furthermore, asbestos heated to 200°C followed by irradiation with 4 MeV X-rays (4500 rads) resulted in reactivation of asbestos cytotoxicity. Scanning electron microscopy indicated that the ratios of free to fiber-associated alveolar macrophages and the fiber fragment size distributions were unaffected by either heat pretreatment or X-ray irradiation. These observations strongly suggest that the surface charge characteristics and electronic state of asbestos fibers may be responsible for its biological activity.

Introduction

Numerous investigations have attempted to correlate the physical and chemical characteristics of asbestos with its associated biological toxicity (1-3). While it has been suggested (4-6) that the physical dimensions of asbestos (i.e., surface area, fiber length, diameter and aspect ratio) may affect its carcinogenicity in rats after intrapleural injection, it remains uncertain whether fiber size alone determines the carcinogenic potential. Recent findings, for example, indicate that the surface charge properties of asbestos play a role in its biological activity (7, 8).

In this regard, modifications to the charge characteristics of chrysotile asbestos, including pretreatment with acids (9, 10) or polyanionic compounds (11, 12) decrease the cytotoxicity of asbestos fibers *in vitro.* Furthermore, studies on rabbit alveolar macrophages (8) and red blood cells (13) indicate that heat pretreatment of asbestos fibers can diminish cellular lysis compared to unheated asbestos.

We have observed that asbestiform minerals exhibit characteristic and unique temperature-dependent luminescence (14, 15). These "glow curves" (Fig. 1) have their physical basis in the thermally induced release of electrons trapped within asbestos lattice imperfections. Such temperature-dependent phenomena may modify the surface charge properties of asbestos fibers and alter cytotoxicity. This hypothesis was evaluated by studying the cytotoxic response of heat-treated and X-ray-irradiated chrysotile asbestos on cultured bovine alveolar macrophages and normal human fibroblasts, two potential cellular targets of asbestos fibers. Furthermore, molecular interactions of asbestos were evaluated by quantifying bovine serum albumin binding.

*Toxicology and Health Sciences Section, Battelle, Columbus Laboratories, 505 King Avenue, Columbus, OH 43201.

†Department of Health and Human Services, Food and Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079.

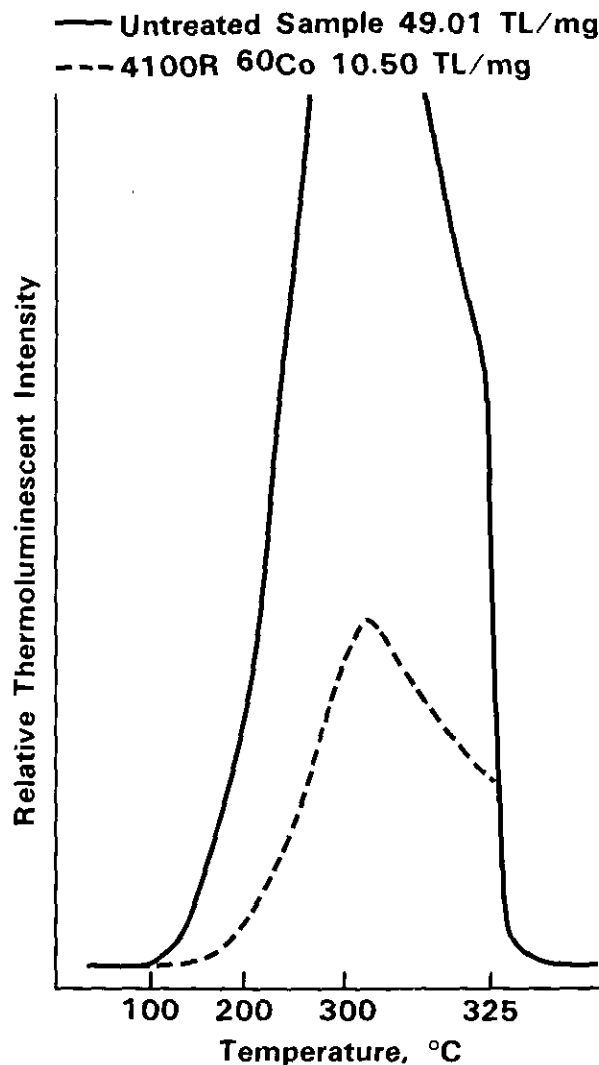‡Donner Laboratory, University of California, Berkeley, CA 94720.

FIGURE 1. Typical "glow curve" representing the relative thermoluminescence obtained upon heating samples of UICC Canadian chrysotile asbestos: (— —) thermoluminescent profile generated after irradiation of the previously heated sample with 4100 rads γ-radiation (^{60}Co source). Details of this technique are described elsewhere (14, 15).

Materials and Methods

Asbestos Preparation

Two different standardized sources of chrysotile asbestos were used in these studies. Table 1 lists several physical properties of Plastibest-20 (National Institute of Environmental Health Sciences, Washington, D.C.) and UICC 15-5 (Particle Information Services, Grants Pass, OR) Canadian chrysotile asbestos standards. Ultraviolet (UV) light, X-ray irradiation, acid or heat pretreatment of 0.5-1 mg asbestos samples was performed in clean, uncapped

glass vials. Heat pretreatment of asbestos was conducted at 70, 100, 200 or 400°C for 3-5 hr. In order to minimize the possibility of bacterial contamination, some asbestos samples were irradiated with ultraviolet light for 3 hr (254 nm, 1.2 mW/cm²). Acid pretreated asbestos was prepared by washing 1 mg asbestos samples in 10 mL of 1 M HCl for 48 hr at 25°C. Asbestos fibers, either unmodified or heat-pretreated at 200°C for 3 hr, were also exposed to energetic radiation. In these studies, 1 mg asbestos samples were irradiated with a 4 MeV X-ray beam from a Varian Clinac-4 linear accelerator. Approximately 4500 rads were delivered to the asbestos as determined by LiF thermoluminescent dosimetry. After all treatments, the asbestos fibers were washed once with phosphate-buffered saline (PBS), centrifuged at 1500g for 15 min and then resuspended in distilled water or PBS before use.

Bovine Serum Albumin Adsorption

Bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO), prepared in distilled water, was added to three replicate samples of heat-pretreated chrysotile asbestos at a 1:10 BSA/asbestos weight ratio (16). Upon addition of BSA, the samples were capped, vortexed and stored for 41 days at room temperature. Thereafter, the samples were centrifuged at 1000g for 5 min, and the supernatant was analyzed for protein by a commercial dye-binding assay (17). Control vials containing BSA without asbestos were analyzed simultaneously.

Bovine Alveolar Macrophage Toxicity Assay

Interactions involving asbestos and mammalian

Table 1. Physical characteristics of UICC and NIEHS chrysotile asbestos.[a]

	NIEHS	UICC
Specific surface area, m²/g[b]	24.9 ± 2.2	26.8 ± 0.7
Density, g/cm³	2.61 ± 0.02	2.4 – 2.6
Mean fiber length, μm	21[c]	6[d]
Elemental composition:		
Fe, wt-%	2.0	2.6
Al, wt-%	0.8	0.27
Mg, wt-%	24.2	32
SiO₂, wt-%	39.9	38.6
Co, ppm[e]	110	46
Cr, ppm[e]	400	490
Ni, ppm[e]	500	490
Mn, ppm[e]	500	510

[a]Data Technical Information Brochure, Particle Information Services, Grants Pass, OR and Bureau of Mines report RI 8452 (1980).
[b]Determined by nitrogen adsorption.
[c]Measured by scanning electron microscopy.
[d]Measured by light microscopy.
[e]By neutron activation.

lung cells were examined by using cultured bovine pulmonary alveolar macrophages (BAM). BAM were isolated by lavage of adult steer lung lobes (*18*). Lung lavage was performed repeatedly with 60-mL aliquots of ice-cold Ca^{2+}- and Mg^{2+}-free PBS, until a total of 400 mL of lavage fluid had been collected. The cell pellet obtained after low-speed centrifugation (300*g*, 30 min) was resuspended in Dulbecco's modified Eagle's medium (DME) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2.5 μg/mL fungizone, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.3 mg/mL L-glutamine (GIBCO, Grand Island, NY). Aliquots of the cell suspension were removed for assessment of viability (trypan blue exclusion), total cell yield (hemacytometer counting) and differential cell counts (*19*). Cellular composition of lung washouts was typically 90% BAM, 8% lymphocytes and 2% neutrophils. BAM viability usually exceeded 90%. After the initial attachment and incubation period, cultures were almost exclusively BAM (≥97%).

Leighton tubes, fitted with 10.5 × 35 mm glass coverslips, were seeded with 10^6 viable BAM and allowed to attach in a humidified, 5% CO_2-95% air incubator. Following a 4-hr attachment period, medium containing nonadherent cells was removed and replaced with fresh medium. After overnight incubation, BAM cultures were washed three times with 1 mL of DME media containing 0.2% hydrolyzed lactalbumin (LH-DME, GIBCO) immediately prior to the introduction of asbestos.

Suspensions of UICC standard 15-5 asbestos fibers (sonicated briefly in LH-DME) or media controls were added to BAM monolayers. Incubation continued for an additional 20-44 hr before the adherent cell viability was determined by light microscopy. Cytotoxicity data were analyzed from triplicate BAM cultures in separate experiments using different BAM sources. Student's *t*-test and regression analysis were used for data comparison with the significance level set at $p < 0.05$ (*20*).

Fibroblast Toxicity Assay

Normal human fibroblasts (human foreskin, line 149) were cultured at the 16th or 17th passage in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum (GIBCO) (*21*). Confluent fibroblast monolayers were harvested by trypsinization (0.01% trypsin in 0.01% EDTA) and dispersed in growth medium to achieve a single-cell suspension. One million fibroblasts were seeded into 100-mm diameter Petri dishes 24 hr before asbestos exposure. Cell cultures were washed twice with PBS, followed by the addition of 10 mL of serum-free medium (Eagle's minimum essential medium,

GIBCO) containing 10 μg/mL NIEHS chrysotile asbestos. To minimize bacterial contamination, control asbestos samples were heated at 70°C for 5 hr. Fibroblast growth inhibition and cytotoxicity was assessed microscopically in triplicate cultures by trypan blue dye exclusion.

Scanning Electron Microscopy

A scanning electron microscope (SEM) was used to examine asbestos fibers after pretreatment and to evaluate BAM interactions with asbestos. UICC asbestos fibers from the four treatment groups, i.e., untreated, heat-treated at 200°C, X-ray-irradiated, and X-ray-irradiated plus heat-treated, were prepared for SEM after being dispersed in distilled water, briefly sonicated and deposited onto a 0.4 μm Nuclepore filter (Nuclepore Corp., Pleasanton, CA). Specimens were examined in an AMRay 1000 A scanning electron microscope at 20 kV accelerating voltage following application of a conductive platinum coating to the fibers. Fiber size classification was performed at 10,000×, by using a diffraction grating for SEM magnification calibration.

To study BAM interaction with asbestos, BAM were fixed on glass coverslips in gluteraldehyde, dehydrated with ethanol and critical point dried (*22*). Macrophages exposed to the four asbestos treatment groups mentioned above were examined at two levels (0.01 and 0.02 mg/mL medium) 44 hr after addition to BAM cultures. In addition to characterizing the surface features of BAM, the percentage of macrophages physically associated with long (> 20 μm) fibers versus free macrophages was quantified from low magnification micrographs.

Results
Macrophage Studies

Initially, to evaluate the requirement for asbestos sterilization, the effects of ultraviolet irradiation on asbestos toxicity was assessed at several concentrations of untreated and ultraviolet-irradiated UICC chrysotile asbestos. At 20 hr after fiber challenge, exposure to either untreated or ultraviolet-irradiated asbestos caused a dose-dependent decrease in attached cell viability (Table 2). There were no significant differences in viability between groups when equal doses were compared. Because viability testing did not detect differences between untreated and ultraviolet-irradiated asbestos and to avoid bacterial contamination, ultraviolet-irradiated asbestos was subsequently used as the positive control to assess the effects of heat treatment.

BAM viability was evaluated 44 hr after the addition of ultraviolet-irradiated, heat-treated (200°C

Table 2. Cytotoxicity comparison of ultraviolet-irradiated
and unirradiated UICC asbestos.

Added asbestos, mg/mL of media	Viability, %[a]	
	Untreated	UV[b]
0	95.8 ± 1.0	
0.001	90.1 ± 2.8	89.6 ± 2.9
0.005	81.6 ± 3.3	80.1 ± 2.0
0.020	57.2 ± 5.0	59.1 ± 6.4

[a]Data expressed as percent bovine alveolar macrophage
viability ± standard deviation from triplicate cultures.

[b]Irradiated asbestos with ultraviolet light (254 nm, 3 hr at
1.2 mW/cm²).

and 400°C) and acid-leached chrysotile asbestos (Fig.
2). Heat pretreatment and acid leaching signifi-
cantly reduced toxicity to BAM cultures relative to
control asbestos. Slopes obtained by regression
analyses of the viability data indicated no differ-
ences between either heat treatment and acid leach-
ing. To evaluate the temporal stability of the modi-
fied asbestos, samples were retested for BAM cyto-
toxicity after remaining in LH-DME media for 5
days. No change in relative cytotoxicity was ob-
served; however, the low dose ultraviolet-irradiated

asbestos control was significantly less cytotoxic
than the same sample tested 5 days earlier.

The influence of X-ray irradiation (4500 rads) on
both unmodified and heat-pretreated asbestos
(200°C, 3 hr) toward BAM cytotoxicity was also
evaluated. Macrophage viability was at least 30%
greater at levels of 0.02, 0.04 and 0.06 mg/mL from
the heat-treated group compared to either the X-
ray-irradiated or heated and X-ray-irradiated asbes-
tos samples. The control samples reduced viability
to intermediate values distinct from the other
groups (Fig. 3).

Fibroblast Studies

The acute cytotoxicity of chrysotile asbestos was
also determined with cultured normal human fibro-
blasts. Cytotoxicity was measured as fibroblast
growth inhibition 24 hr after exposure to 10 μg/mL
of heat-treated asbestos. At the three temperature
levels studied, increasing the asbestos pretreatment
temperature reduced growth inhibition in fibroblast
cultures (Table 3). The relative relationship of cell
growth inhibition was consistently observed to be
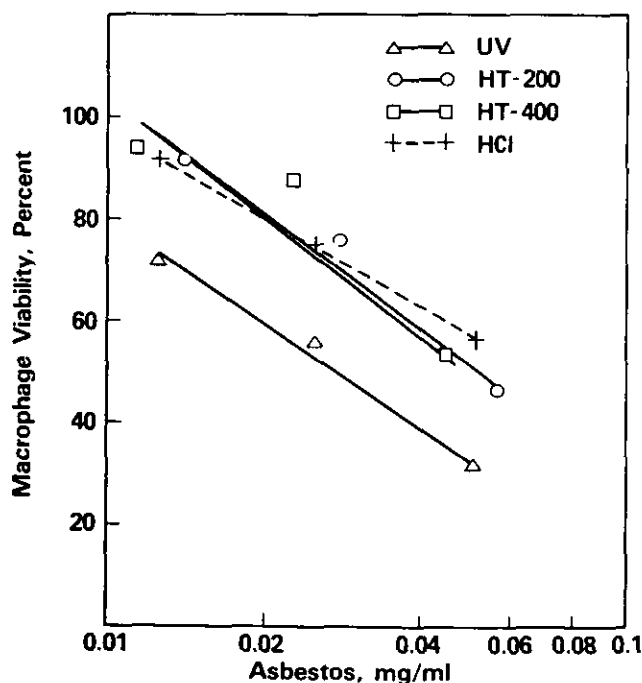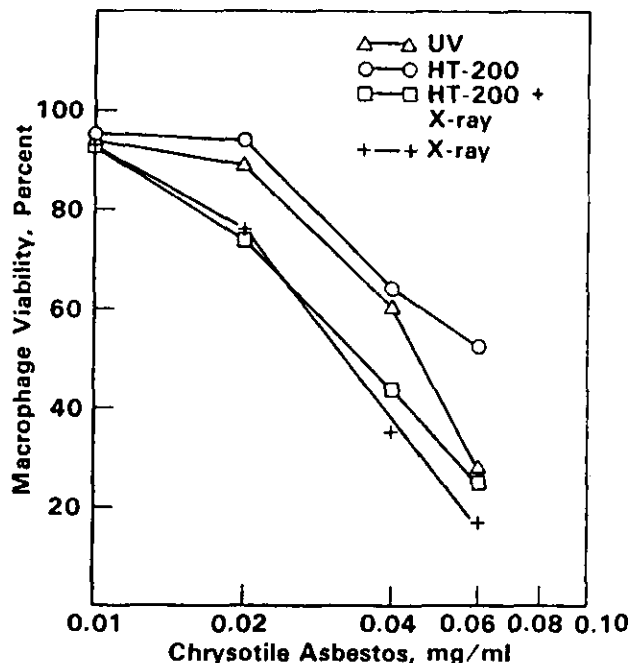70°C > 200°C > 400° C = unexposed fibroblast con-

FIGURE 2. Bovine alveolar macrophage viability following ex-
posure to selected levels of UICC chrysotile asbestos.
Viability was assessed 44 hr after introduction of asbestos.
Treatments depicted are: UV, irradiated with ultraviolet
light, 254 nm; HT-200, heat-treated asbestos, 200°C for 3
hr; HT 400, heat-treated asbestos, 400°C for 3 hr; and HCl,
1 N HCl-leached for 48 hr at 25°C.

FIGURE 3. Bovine alveolar macrophage viability following in
vitro exposure to heated and X-ray-irradiated UICC chrys-
otile asbestos. Viability was assessed 44 hr after introduc-
tion of asbestos. Treatments depicted are: UV, irradiated
with ultraviolet light, 254 nm; HT-200, heated-treated
asbestos, 200°C for 3 hr; HT-200 + X-ray, heat-treated
asbestos, 200°C for 3 hr, followed by X-ray irradiation, 4
MeV, 4500 rads; X-ray, X-ray irradiated asbestos, 4 MeV,
4500 rads.

Table 3. Effect of chrysotile asbestos heat pre-treatment on bovine serum albumin binding and fibroblast growth.

Asbestos	Pretreatment temperature, °C	Bound BSA, μg BSA/mg asbestos	Fibroblast growth, cells/plate $\times$ 10^{-3a}
Untreated control	--	--	12.4 ± 0.7
NIEHS	70	73.7 ± 3.8	9.8 ± 0.5[b]
	100	74.7 ± 0.7	c
	200	73.9 ± 2.9	11.0 ± 0.8[b]
	400	53.4 ± 8.3[b]	13.0 ± 0.8
UICC	70	74.7 ± 2.1	c
	400	56	c

[a]One million normal human foreskin fibroblasts were treated with 10 μg/mL chrysotile asbestos for 24 hr in serum-free media. Data are expressed as mean ± standard deviation from triplicate cultures.
[b]Statistically significant difference ($p < 0.05$) from controls.
[c]Not performed.

trol. Thermal pretreatment of asbestos to 400°C decreased the acute cytotoxicity of chrysotile fibers by approximately 25%

The relative adsorptive capacity of heat-treated chrysotile asbestos for bovine serum albumin was also examined. The adsorption of BSA by thermally modified chrysotile at 100 or 200°C was not significantly different from the control asbestos samples (Table 3). However, either UICC or NIEHS chrysotile asbestos adsorbed approximately 25% less BSA than control samples when heated to 400°C.

Scanning Electron Microscopy

SEM examination of UICC chrysotile asbestos revealed many long, coiled fibers with fibrous sub-
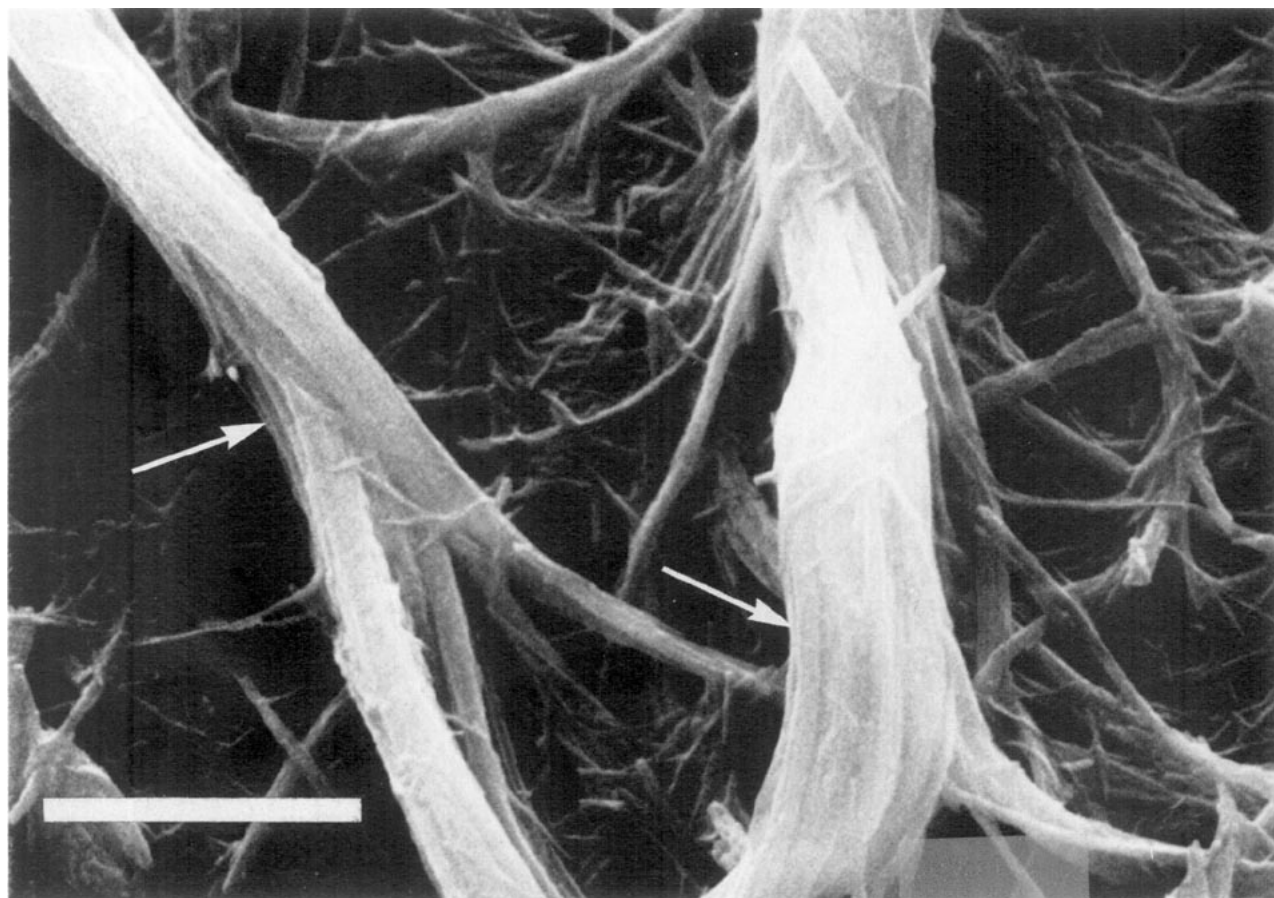

FIGURE 4. Scanning electron micrograph of untreated chrysotile asbestos fibers. The long, twisted appearance of fibers is evident and shows that the fibers are composed of fibrous subunits (arrows), 9,700×, bar = 1μm).
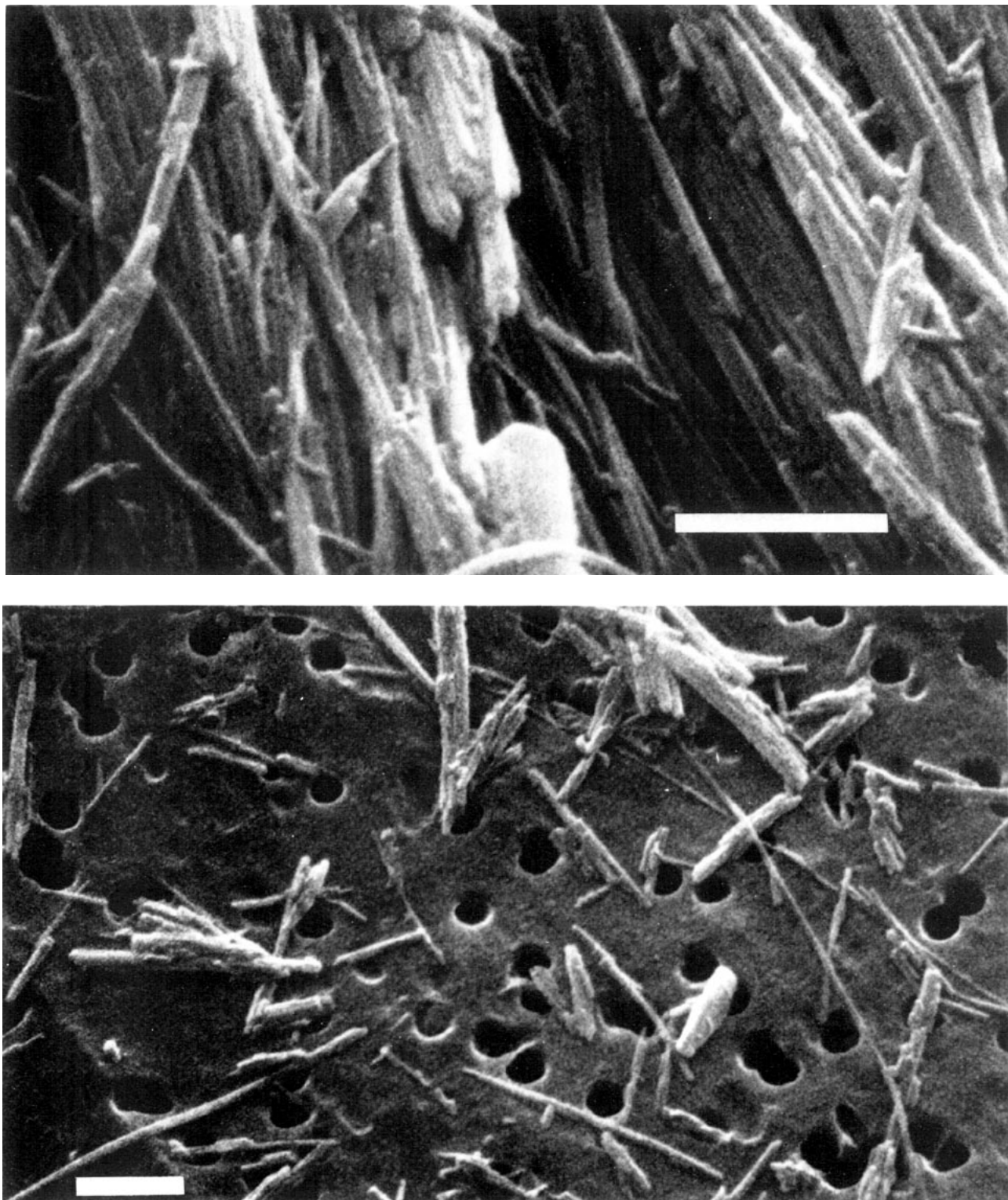
FIGURE 5.   High magnification scanning electron micrographs: (A) X-ray-irradiated asbestos showing fiber fragments breaking off from a large fiber (20,000 ×, bar = 1 μm); (B) fiber fragments, visible on the Nuclepore filter (9,700 ×, bar = 1μm) were measured for fiber size distributions.

units and numerous fiber fragments in all samples (Fig. 4). Small fragments were often found in the process of breaking off from larger fibers and were frequently observed on the filter (Fig. 5A). It was extremely difficult to obtain useful fiber size information from low magnification micrographs containing fiber clusters due to the inability to distinguish individual fibers. Therefore, we decided to size classify individual fiber fragments at 10,000× magnification from micrographs similar to Figure 5B. Between 43 and 74 fiber fragments per asbestos sample were measured for length and width. The fiber size distribution appeared logarithmically normal for all samples and no significant differences were observed between the count median lengths for each asbestos treatment (Table 4). Similarly, no differences were observed between fiber widths or length:width ratios. Fiber fragments widths were normally distributed with a mean value of 0.12 μm for all samples.

Bovine alveolar macrophage interactions with asbestos were examined for each of the four asbestos treatments. Between 200 and 500 cells per sample were counted to determine the percentage of BAM associated with long fibers (greater than 20 μm) compared to unassociated BAM. No significant differences were observed in the ratio of free to fiber-associated BAM for the various fiber treatments. However, a significant dose-dependent increase in the number of macrophages attached to fibers was observed, since 39% and 74% of BAM were associated with long fibers for the low and high doses, respectively.

Analyses of SEM stereo pair images suggested that three morphological classes of macrophage-asbestos fiber interactions were observed. The two most common classes were composed of elongated macrophages stretched along fibers with predominantly smooth surfaces and adjacent, rounded macrophages displaying various surface features (Fig. 6A). Cell morphology at the cell/fiber interface was variously characterized by the presence of smooth surfaces, ruffles and/or blebs (Figs. 6B, C, D). The

third morphological class consisted of relatively few BAM (< 3% in all samples) that exhibited a loss of membrane integrity, typified by numerous holes and a lack of surface features (Fig. 7). This mottled appearance has been observed after Ni_3S_2 exposure (unpublished observation) and exposure to quartz (23). In all classes, small fibers and fiber fragments were not seen on the coverslips and were probably internalized by BAM after the 44-hr culture period.

Discussion

These studies indicate that chrysotile asbestos is acutely cytotoxic to bovine alveolar macrophages and human fibroblasts using serum-free media *in vitro*. A significant decrease was observed in cellular viability with increasing concentrations of untreated asbestos fibers. In contrast to untreated asbestos, comparable levels of either 200 or 400°C heat-pretreated chrysotile asbestos were significantly less cytotoxic to cultured human fibroblasts or BAM. The observation that heat pretreatment ameliorates chrysotile asbestos cytotoxicity toward macrophages was also reported by Robock and Klosterkotter (8). They noted that oxygen consumption, used as an index of macrophage viability, was markedly depressed in cultures exposed to asbestos heated to 200°C. However, an equal concentration of asbestos previously heated to 400°C resulted in oxygen consumption levels identical with unexposed, control macrophages. They noted that with pretreatment temperatures in the range of 550-650°C, chrysotile asbestos loses crystalline water to form a new minerals species, forsterite, which is more cytotoxic than the parent chrysotile (8, 13).

The relationship between the solubility of magnesium and the biological activity of asbestos has been well described. In general, cytotoxicity is inversely related to the magnesium content of the untreated fibers and to the magnitude of its associated positive charge on fiber surfaces (9, 10, 24). With acid leaching, however, the exposed layer of magnesium is dissolved, markedly reducing the positive surface charge and its hemolytic and cytotoxic effects (7, 12, 24). Although we selected only one time period for the HCl leaching study, our data demonstrated the cytotoxic equivalency of the acid-leached and heat-treated asbestos groups. While more extensive leaching in 1 N HCl may further reduce BAM cytotoxicity, some preliminary evidence was obtained to suggest that cytotoxicity is also reduced after leaching at physiological pH in serum-free media. Since the reduction in cytotoxicity was observed only in the low-dose ultraviolet-irradiated asbestos group, further studies are required to confirm this observa-

Table 4. Fiber fragment length distributions of pretreated UICC chrysotile asbestos.[a]

Asbestos pretreatment	Count median length, μm	Geometric standard deviation	n^{b}
Untreated	0.71	1.80	43
HT-200	0.64	1.81	47
X-ray	0.56	1.94	74
HT-200 + x-ray	0.63	1.64	70

[a]Treatments depicted are: HT-200, heat-treated at 200°C for 3 hr; X-ray, irradiated with 4 MeV X-rays, approximately 4500 rads.

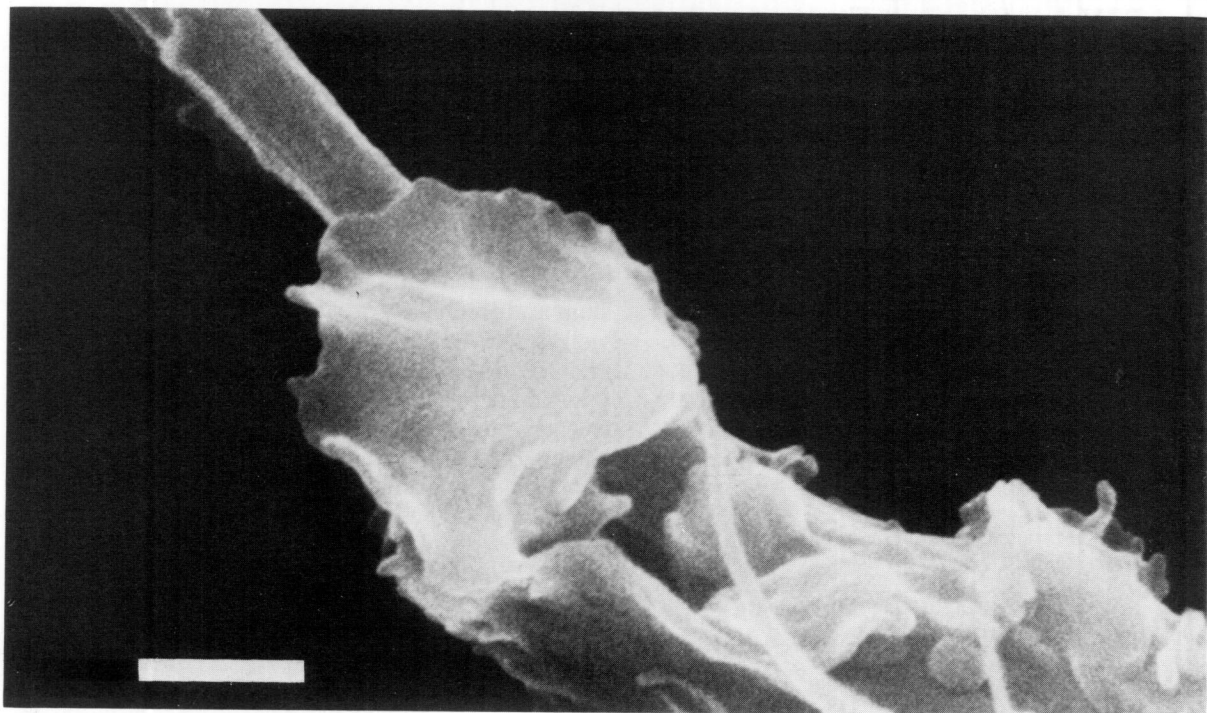[b]Number of fibers counted per treatment.
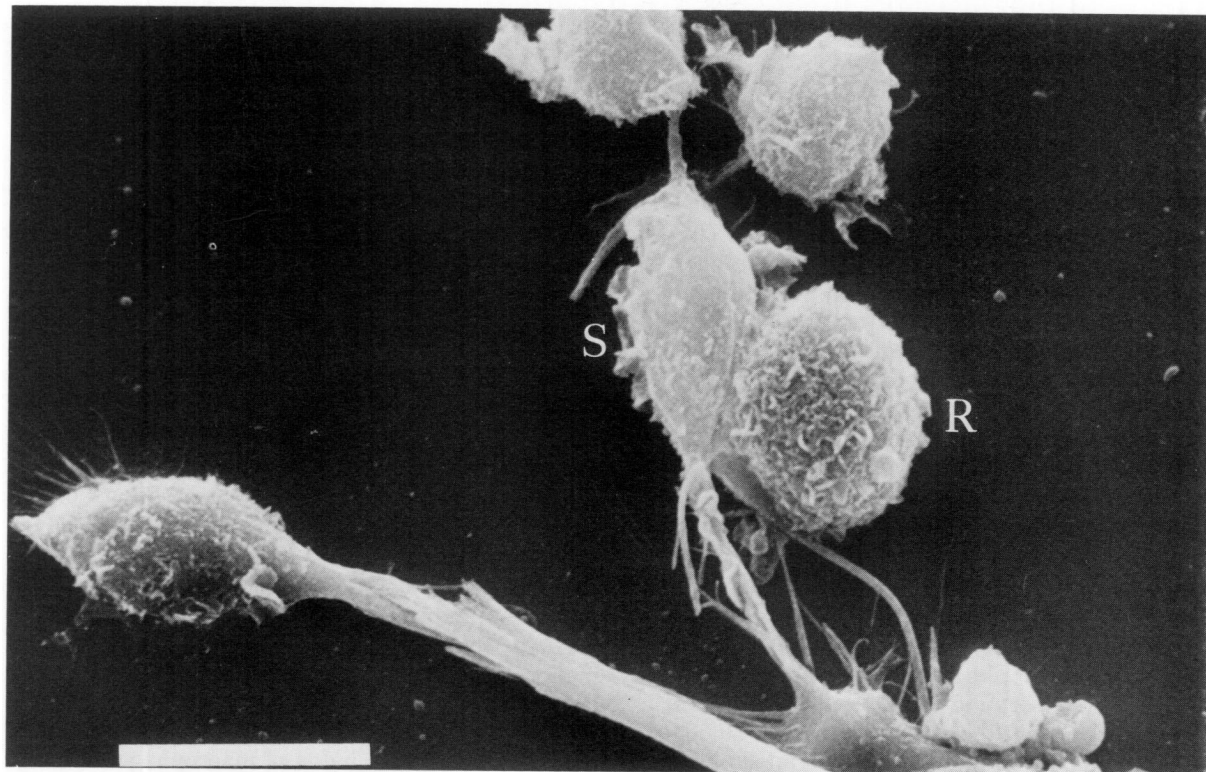
FIGURE 6.   Scanning electron micrographs of untreated UICC chrysotile asbestos fibers associated with bovine alveolar macro-
phages: (A) cells(S) were often observed spread along asbestos fibers with additional rounded macrophages (R) nearby (1940×,
bar = 10 μm). Structures such as ruffles (B) (11,640×, bar = 1 μm), blebs (C) (19,400×, bar = 1 μm) and smooth surfaces (D)
(20,000×, bar 1 μm) were commonly observed in macrophages from each of the asbestos treatment groups.
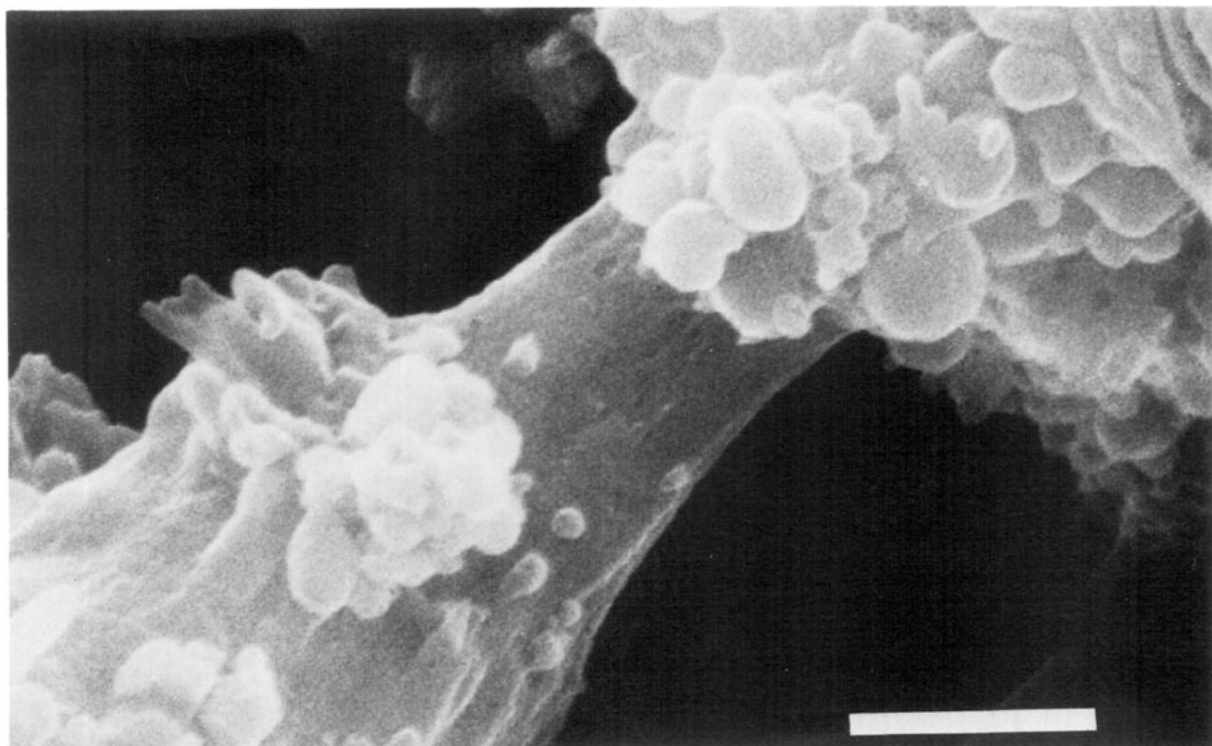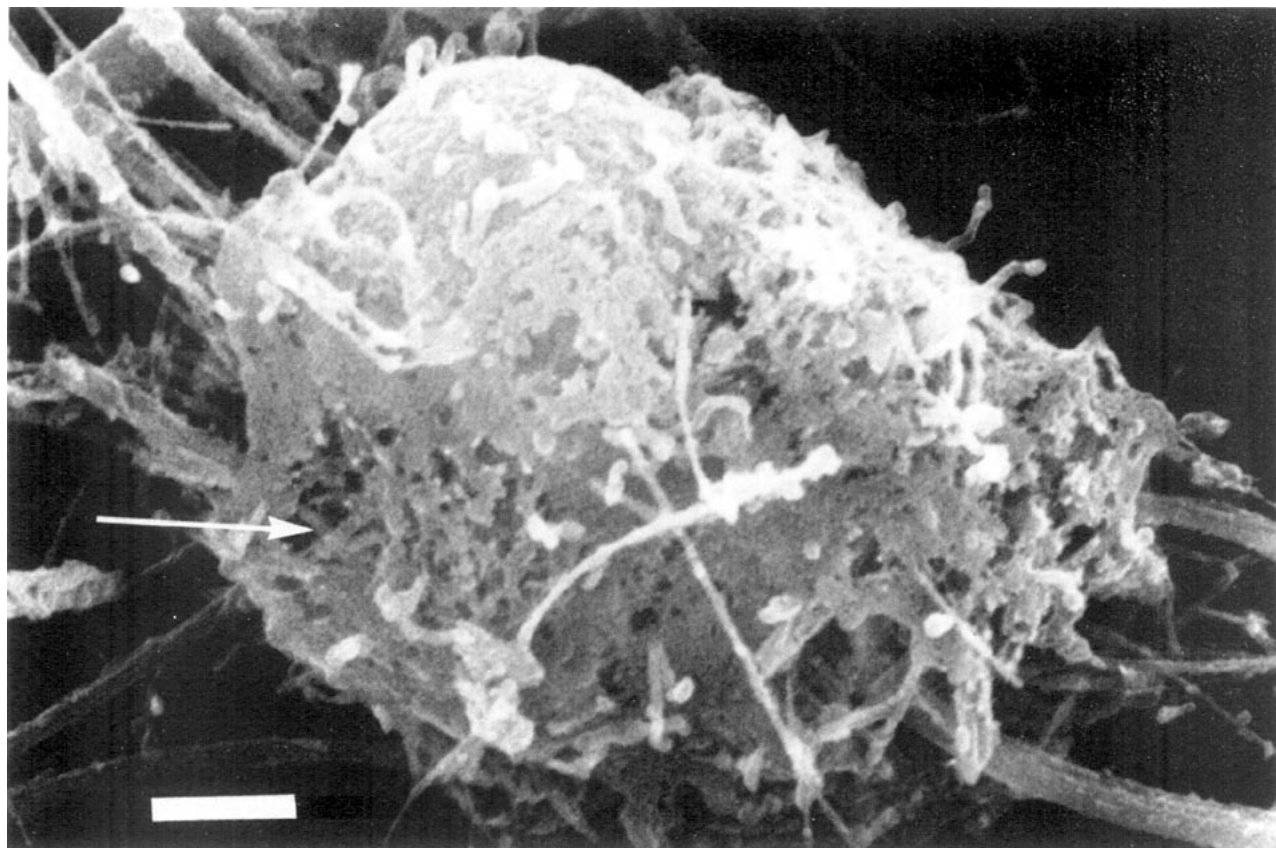
(C)

(D)

FIGURE 7. Some macrophages appeared mottled with numerous holes and a loss of typical membrane surface features. Fibers, previously X-ray irradiated, are visible (arrow) on such cells (9,700×, bar = 1 μm).

tion. Although a correlation may be established between cytotoxicity and magnesium in asbestos, it is unclear whether magnesium is directly involved in reducing the cytotoxic and thermoluminescent profiles of asbestos after heat pretreatment.

Several investigators have studied the charge-related interactions between asbestos and organic compounds (11, 12, 25) or macromolecules (24, 26). Our data indicate that heat treatment of chrysotile asbestos decreases the positive surface charge based on its affinity for the anionic macromolecule, albumin.

Morgan (24) has observed that BSA binding is proportional to both the total available surface area and magnesium content of chrysotile asbestos fibers. Accordingly, the degree of fiber fragmentation or magnesium depletion may govern the amount of BSA bound to chrysotile. However, our results demonstrate that, although the mean fiber sizes of NIEHS and UICC chrysotile are different, comparable levels of BSA binding were observed, both before and after asbestos heat pretreatment. Conceivably, fiber surface charge may be of greater importance as a determinant of BSA binding than

fiber size. Indeed, Morgan (24) has shown that with increased magnesium depletion, BSA binding decreases even though asbestos surface area increases (27). Further, our observation that BSA binding was reduced only at 400°C may be indicative of the relative insensitivity of the BSA binding technique in comparison to the cytotoxicity assays for detecting alterations in fiber surface charge.

Notably, our studies indicate that X-ray irradiation of a previously heated asbestos sample is more cytotoxic to bovine alveolar macrophages than either the ultraviolet-irradiated control or heat-pretreated asbestos samples. This effect is independent of an X-ray-induced alteration in the susceptibility of asbestos to macrophage phagocytosis, since similar numbers of fibers were associated with BAM from all treatment groups. Additionally, SEM analyses failed to demonstrate morphological alterations to chrysotile asbestos or BAM associated asbestos, regardless of fiber pretreatment.

These observations provide the basis for a hypothesis on asbestos fiber cytotoxicity. Accordingly, the crystalline properties of asbestos result in localized electronic charge centers which may partici-

pate in electron transfer reactions. The presence of crystal lattice defects within asbestos fibers permits electron transfer with cellular membranes (8, 28-30).

Because naturally occurring asbestiform minerals contain lattice imperfections (through impurity incorporation or lattice vacancies), localized charged centers or electron traps may result within the crystal structure. Energetic particles or ionizing radiation striking the crystal will excite or displace electrons from their low energy ground state (valence band) to a higher energy state in the conduction band. Some of the excited electrons rapidly return to the ground state, with the subsequent release of electromagnetic radiation (radioluminescence). Other electrons, however, may migrate into electron "traps" created by crystal lattice defects at energy levels below the conduction band. If the electron traps occur at energy levels closely matched to those in biological materials, they may participate in electron transfer reactions (29, 31). The trapped electrons, however, may also return to their ground state if the crystal is heated (32). The additional thermal energy releases the trapped electrons to the ground state with the concurrent emission of light (thermoluminescence). In this respect, we have observed that many asbestiform minerals are thermoluminescent and that irradiation of heated asbestos regenerates the thermoluminescent pattern of thermally discharged asbestos minerals. These observed physical processes correlate well with the changes in cytotoxicity reported here.

Our data are consistent with the hypothesis that the electronic state modifies *in vitro* asbestos cytotoxicity. Indeed, the observations on the uniform fiber size distributions of the treated asbestos samples, the qualitative similarity of fiber phagocytosis among the asbestos treatment groups *in vitro*, the decrease in cytotoxicity following heating and the recovery of cytotoxic activity after energetic irradiation indicate that trapped electrons participate in asbestos fiber-biomembrane interactions.

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